Miltenyi Biotec



MACSQuant® Tyto® Instrument short instructions

Compensation

These short instructions are valid for MACSQuantify™ Tyto Software 3.2 and later.

Multiparameter cell analysis allows the examination of multiple cellular properties by simultaneously detecting different fluorochromes that identify these properties. Due to fluorescence spectral overlap or spillover, individual fluorochromes are detected in more than one fluorescence channel. Thus, for multicolor analysis, compensation of spectral overlap is necessary to produce accurate and consistent results.

Automated compensation

Prerequisites for automated compensation

Follow the general compensation guidelines. See **Compensation guidelines** in the MACSQuantify Tyto Software 3.2 user manual. Consider following prerequisites for successfull automated compensation.

- Fill the mixed compensation control sample into the cartridge and start the automated compensation immediately. Do not store the mixed sample before starting the compensation.
- The MFI of the positive population must be at least 8.
- If using other material than MACS Comp Beads, prepare a sample containing at least 3×10^5 events/mL in total.
- The region of interest (ROI) must have at least 500 positive events in the respective target channels.
- It is recommended to provide at least 500 µL compensation control sample in the cartridge.

Materials required

- MACSQuant Tyto Cartridge
- MACSQuant Running Buffer
- 1.5 mL sample tubes
- Centrifuge
- Fluorochrome-conjugated antibodies used in the experiment
- MACS Comp Beads suitable for the used fluorochrome-conjugated antibodies (for example, MACS Comp Bead Kit, anti-REA, # 130-104-693)

Preparation of MACS Comp Beads

1 Label a separate sample tube for each fluorochrome-conjugated antibody used in the experiment.
These are the single stain controls.

2 Label one additional sample tube for the blank beads.

3 Add 150 µL of the MACSQuant Running Buffer to each single stain control sample tube.

4 Vortex the MACS Comp Beads and the MACS Comp Beads – blank thoroughly before use.

5 Add one full drop of MACS Comp Beads per single stain control.

6 Add the appropriate amount of fluorochrome-conjugated antibodies to the appropriate sample tube. For an antibody dilution of 1:50, use 4 µL antibody.

7 Mix well and incubate for 5 to 10 minutes in the dark at room temperature. Shake sample during incubation.

8 Prepare the blank sample during incubation. Add 250 µL of the MACSQuant Running Buffer to the blank sample tube and add one full drop of the MACS Comp Beads – blank.
The blank sample does not require washing.

9 To wash the single stain controls, add 1 mL of MACSQuant Running Buffer to each single stain control sample and mix well.

10 Centrifuge the single stain control sample tubes at 300×g for 5 minutes at room temperature.

11 Carefully aspirate or decant the supernatant.

12 Resuspend each pellet in 300 µL MACSQuant Running Buffer.

13 Ensure that the MACSQuant Tyto Instrument is calibrated and the workspace is set up. See **Protocol for automated compensation**, steps **1**–**5**.

14 Load the single stain control samples and the blank sample into the cartridge. Load equal volumes of the single stain control samples.

15 Proceed immediately with the autocompensation. See **Protocol for automated compensation** steps **6**–**13**. Do not store the mixed compensation control sample.

Protocol for automated compensation

1 Ensure that the MACSQuant Tyto Instrument is calibrated.

2 Go to **File > New workspace** to open an empty workspace.

3 Optional: Open an existing workspace containing the correct PMT channel values. Refer to the **MACSQuant Tyto instrument short instructions Setting up a sort workspace**.

4 Adjust all PMT channels. Make sure that each fluorescent signal is the brightest signal in its designated channel. See **Setting fluorescence channel gains** in the MACSQuantify Tyto Software 3.2 user manual.

5 Adjust the sample volume in the **Experiment** tab according to the provided volume in the cartridge.

6 Scan and insert the cartridge containing the compensation control samples.

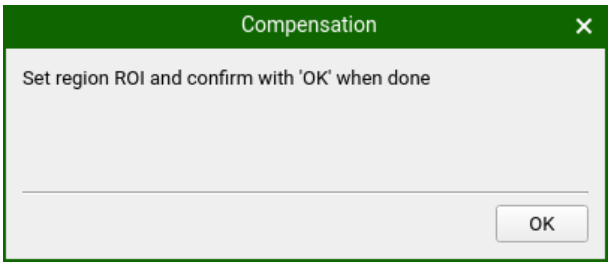
7 Go to the **Compensation** tab on the touchscreen.

8 Select the **Activate compensation** checkbox.

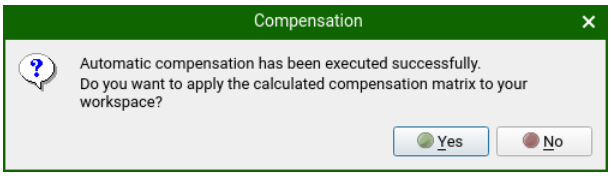
9 Click the **Auto Compensation** button.

10 Follow the instructions in the dialog box.

11 When prompted, adjust the region around the scatter population in the BSV-H/BSB-H plot that defines the cells or beads used for compensation. If working with cells, include only cells which are relevant for the sorting strategy in the ROI.



12 When compensation is completed, a dialog box opens to apply the calculated spillover matrix to your workspace. Click **Yes**.



13 Another dialog box opens. Click **Yes** to save the workspace.

Spillover matrix

The MACSQuantify Tyto Software uses a spillover matrix, that organizes the detection of individual fluorescence signals in columns. Column headers identify the fluorochromes detected on the MACSQuant Tyto Instrument. Rows indicate the fluorescence channels. The default value is set to 1.0 in the diagonal (column versus row). This position within the matrix indicates the primary detection channel for the respective fluorochrome.

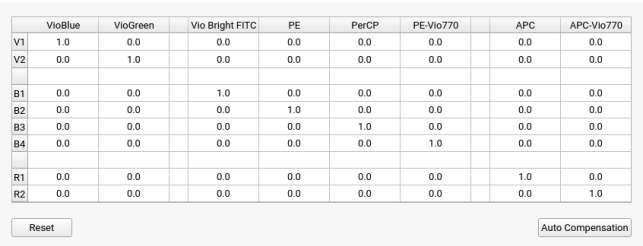


Figure 1: The spillover matrix of the MACSQuant Tyto Cell Sorter on the touchscreen

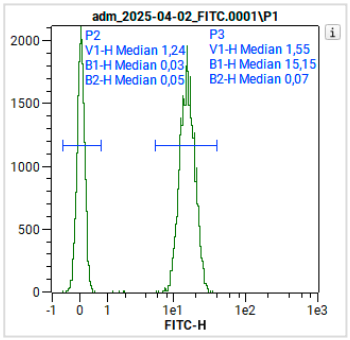
Manual compensation

Materials required

- MACSQuant Tyto Cartridge
- MACSQuant Running Buffer
- Single-stained MACS Comp Beads representing all fluorochromes used in the experimental staining panel. Prepare the MACS Comp Beads according to the data sheet.

Protocol for manual compensation

1 Ensure that the MACSQuant Tyto Instrument is calibrated.
2 Go to **File > New workspace** to open an empty workspace.
3 Optional: Open an existing workspace containing the correct PMT channel values. Refer to the **MACSQuant Tyto instrument short instructions Setting up a sort workspace**.
4 Adjust all PMT channels. Make sure that each fluorescent signal is the brightest signal in its designated channel. See **Setting fluorescence channel gains** in the MACSQuantify Tyto Software 3.2 user manual.
5 To select an analysis template, click the **New analysis window** button.
6 Click the **'i'** button of a plot.
7 Change the plot into a histogram.
8 Select the respective channel of the compensation control sample on the x-axis.
9 Click the **Compensation** tab on the touchscreen to open the spillover matrix. Select the **Activate compensation** checkbox.
10 Scan and insert the cartridge containing the compensation control sample.
11 Click the **Start measurement** button.
12 When events start to appear on the plots, draw a region around the population of interest within the scatter dot plot. This is P1.
13 Select P1 from the plot header of the histogram plot to show only events in P1.
14 Draw an interval region around the negative (P2) and the brightest positive population (P3).



15 Click the **'i'** button of the histogram.
16 To simultaneously show the medians for all channels used in the experiment, go to the **Feature Functions** tab. Select the respective channels and select **Median**.
17 Click **OK**.
18 To adjust compensation, select the appropriate cell in the spillover matrix. For example, to correct the spillover of a Vio Bright FITC-stained sample into the PE channel, go to the cell Vio Bright FITC/B2.
19 To adjust the matrix values in increments of 0.01, click the toggle bar.
20 Adjust the values until the median fluorescence in the spillover channel for the positive and negative populations is equal.
21 Once the compensation is adjusted for one fluorochrome, continue with the compensation for all other fluorochromes.
22 When finished, save as a **Workspace** file.

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